VANM83.001APC

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Communi et al.

Group Art Unit ...

Appl. No : 09/077,173

Filed : December 11, 1998

For : RECEPTOR AND NUCLEIC ACID MOLECULE
ENCODING SAID RECEPTOR

Examiner :

DECLARATION OF Dr Marc PARMENTIER,

Assistant Commissioner for Patents

Washington D.C. 20231

Dear Sir:

I, Dr Marc PARMENTIER, declare as follows :

1. I am a designated inventor in the U.S. patent09/077,173.

2. I have been working for several years in the field of
    molecular biology and I have identified various G-coupled
    receptors and polynucleotide molecules encoding said
    receptor, in particular human and other animal receptors.

3. I reviewed the content of the US patent application
    09/077,173 and based upon the data present in said patent
    application, I can state that it is possible for a person
    skilled in the art to identify other receptors which
    present a high homology with the present human receptor and

which are able to bind nucleotide. As an example of a corresponding animal receptor, I present in the enclosed annex data related to corresponding mouse receptors identified by the group of the same inventors and by other groups.

This mouse receptor has been identified by screening at moderate stringency a mouse genomic DNA library with a human $P2Y_4$ probe.

The identification of other corresponding receptors of other animals could be obtained by using techniques well known by the person skilled in the art and described in the literature for many years.

4. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardise the validity of the application and any patent issued thereon.

Respectfully submitted,

Dated:

By:

Marc Parmentier

EXHIBIT A

Molecular cloning and characterization of the mouse P2Y4 nucleotide receptor

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Abstract

To isolate the mouse $P2Y_4$ receptor gene, a mouse genomic library was screened with a human $P2Y_4$ probe. An open reading frame encoding a protein of 361 amino acids was isolated. This protein showed 82% and 95% amino acid identity with the human and the rat $P2Y_4$ receptors, respectively. By reverse transcription and polymerase chain reaction (RT-PCR), the $P2Y_4$ messenger RNA was detected in mouse liver, intestine, stomach, bladder and lung among the 16 mouse tissues tested. In 1321N1 transfected cells, the mouse $P2Y_4$ receptor was equally activated by UTP and ATP, and was antagonized by pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and Reactive Blue 2, and not by suramin. Moreover, when expressed in 1321N1 cells, the rat $P2Y_4$ is also antagonized by PPADS. Thus, when compared in the same expression system, the mouse $P2Y_4$ is closer to the rat ortholog in terms of agonist stimulation, while in terms of antagonist profile, the three $P2Y_4$ receptor orthologs are similar.

Keywords: $P2Y_4$ receptor, ATP, UTP, P2 receptor antagonists, 1321N1 astrocytoma cell line.

1. Introduction

Despite strong conservation in amino acid sequence, human and rat $P2Y_4$ receptors do not exhibit full conservation in terms of agonist specificity. Indeed, the human $P2Y_4$ is activated preferentially by UTP, and ATP behaved like a partial agonist (Communi et al., 1995) or an antagonist (Kennedy et al., 2000), while the rat ortholog of this receptor is equipotently activated by ATP and UTP (Bogdanov et al., 1998, Webb et al., 1998). The human and rat $P2Y_4$ receptors exhibit also different sensitivity to various P2 receptor antagonists: the rank order of potency being pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS)>Reactive Blue 2>>Suramin=0 for the human $P2Y_4$ receptor expressed in 1321N1 cells (Communi et al., 1996) and Reactive Blue 2>>suramin>PPADS=0 for the rat $P2Y_4$ receptor expressed in *Xenopus* oocytes (Bogdanov et al., 1998).

In the present report, we present data on the cloning, the pharmacological characterization and the tissue distribution of the mouse ortholog of the $P2Y_4$.

2. Materials and methods

2.1. Materials

Trypsin was from Flow laboratories (Bioggio, Switzerland). The culture media, the fetal calf serum, G418, Platinum® *Pfx* DNA polymerase, Superscript™ II preamplification system and restriction enzyme were purchased from GIBCO BRL (Merelbeke, Belgium). Taq polymerase was from QIAGEN Inc (Valencia, CA, USA). Myo-D-2-[^{3}H]inositol (17.7 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) were supplied by Amersham (Ghent, Belgium). FuGENE™ 6 Transfection Reagent was from Boehringer Mannheim. pEFIN3 is an expression vector developed by EUROSCREEN (Brussels, Belgium). Dowex AG1X8 (formate form) was from

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Bio-Rad Laboratories (Nazareth Eke, Belgium). ATP and UTP were obtained from Sigma Chemical Co. (St Louis, MO, USA). Suramin, Reactive Blue 2 and pyridoxal-phosphate-6 azophenyl-2',4'-disulphonic acid (PPADS) were from Research Biochemicals (Natick, MA, USA).

2.2. Cloning and sequencing

A mouse genomic 129/SVJ DNA library in Lambda FIX II (Stratagene, La Jolla, CA) was screened with a radiolabelled human P2Y$_4$ cDNA (600 bp located between transmembrane domains 3 and 7) as a probe. Hybridizations were performed at 42°C in 35% formamide, 5 mM EDTA, 6xSSC (sodium chloride/sodium citrate) and 0.25% nonfat dry milk for 16h and the final wash conditions were 0.5xSSC, 0.12% sodium dodecyl sulfate (SDS) at 55°C. The positive clones isolated were sequenced using an Applied Biosystems Model 370A sequencer.

2.3. Cell culture and transfection

The complete receptor coding sequence was amplified using the Platinum® *Pfx* DNA polymerase and specifc mouse P2Y$_4$ primers, and subcloned between the BamHI and SpeI sites of the pEFIN3 expression vector. 3 µg of recombinant plasmid were transfected into 1321N1 astrocytoma cells using the FuGENE™ 6 reagent. Transfected cells were selected with G418 (400 µg/ml) and maintained in DMEM (Dulbecco's modified Eagle's medium) culture medium (10% fetal calf serum, 5% sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B) containing G418 (400 µg/ml). At confluency, cells were trypsinized (1 mg/ml). The 1321N1 cells transfected with the rat P2Y$_4$ receptor were a generous gift from Kendall Harden, University of North Carolina (Kennedy et al., 2000) .

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2.4. Measurement of inositol triphosphate (InsP₃) production

1321N1 cells were seeded in 35 mm Petri dishes in DMEM culture medium (at 2×10^5 cells/dish). The next day they were labeled 24h with [³H]inositol (10 µCi/ml) in 5% fetal calf serum DMEM culture medium. Cells were then rinced twice and placed in KRH (Krebs-Ringer HEPES) buffer (124 mM NaCl; 5 mM KCl; 1.25 mM MgSO₄; 1.45 mMCaCl₂; 1.25 mM KH₂PO₄; 25 mM HEPES pH 7.4 and 8 mM glucose). After 3h, tested agonists were added in this same medium for 30 sec. When tested, antagonists were added 30 min before stimulation. The incubation was stopped by removing medium and addition of 3% ice-cold perchloric acid solution. Inositol phosphates were separated and extracted on Dowex columns as described previously (Communi et al., 1995). The figures were realized using Sigma plot 2.0. The EC₅₀ and IC₅₀ values were determined by curve fitting (Sigma plot version 2.0).

2.5. Reverse transcription and polymerase chain reaction (RT-PCR) analysis

Total mouse RNA was extracted from different BALB/C organs by TCG/phenol/chloroform extraction as described by Chomczynski & Sacchi (1987). 1 µg of total RNA was submitted to reverse transcription using the Superscript™II Preamplification system with random hexamers primers (GIBCO BRL). Briefly, cDNA was synthetized from 1 µg of each total RNA preparation in a 21 µl reaction volume, in the presence of 200 units of Superscript™II reverse transcriptase according to the manufacturer's recommendations. Oligonucleotide amplification primers (22 and 20-mers) were designed from the mouse P2Y₄ sequence: sense primer 5'-AGCCCAAGTTCTGGAGATGGTG-3'; antisense primer 5'-GGTGGTTCCATTGGC ATTGG-3'. PCR was performed on 2 µl of the RT reaction, under the following conditions: 60 sec at 94°C, 60 sec at 61°C, 60 sec at 72°C for 30 cycles. PCR reactions that included each cDNA

synthesis reagent except reverse transcriptase were set up in parallel as control for genomic DNA contamination. Each PCR reaction was performed on two independent RT reactions. Amplification products were resolved on a 1.5% (w/v) agarose gel by electrophoresis.

3. Results

3.1 Cloning of the mouse P2Y4 receptor

Four clones were isolated by the screening of a mouse genomic library with a human $P2Y_4$ probe. Analysis by restriction and Southern blotting revealed that each clone contained an identical 16 kb insert. Subcloned fragments of this insert were sequenced and an intronless open reading frame of 361 codons was identified (accession number AJ277752; Fig. 1). The comparison of deduced amino acid sequences of rat $P2Y_4$ and mouse $P2Y_4$ revealed that they were 95% identical, while human $P2Y_4$ and mouse $P2Y_4$ exhibited 82% of identity in amino acids (Fig. 1).

3.2. Functional characterization of the mouse P2Y4 receptor in the 1321N1 cells

The open reading frame of the $P2Y_4$ was amplified by PCR reaction, sequenced and subcloned in the pEFIN3 expression vector. After transfection with the recombinant vector, the 1321N1 astrocytoma cells were selected and characterized for their response to different nucleotides. As shown in Fig. 2, UTP and ATP were equiactive and equipotent on the murine $P2Y_4$ receptor (EC_{50} UTP = 0.4 ± 0.08 μM; EC_{50} ATP = 0.7 ± 0.3 μM). The ability of suramin, PPADS and Reactive Blue 2 to inhibit the UTP response was tested. At 100 μM, suramin had no effect on the $InsP_3$ accumulation induced by 1 μM UTP in transfected cells (data not shown). At the same concentration, PPADS and Reactive Blue 2 inhibited this response by $69.8\pm3.4\%$ and

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60.3±6.6% respectively (data not shown). The potencies of PPADS (IC_{50} = 45±15 µM; Fig. 3a) and Reactive Blue 2 (IC_{50} = 47±12.5 µM; Fig. 3b) were comparable. These antagonists increased the EC_{50} for UTP (Fig. 4a and b). However, this shift in the concentration-effect curve induced by PPADS was acccompanied by a significant decrease in the maximal stimulation of $InsP_3$ produced by UTP (Fig. 4a). This indicated that the inhibitory effect of PPADS on the UTP response does not occur by competitive inhibition.

A different sensitivity for PPADS as been described between the human and the rat $P2Y_4$ receptor orthologs (Communi et al., 1996; Bogdanov et al., 1998). However, these two receptors were not compared in the same expression system. We observed here that, when expressed in 1321N1 cells, the rat $P2Y_4$ is antagonized by PPADS with a similar potency as the human and the mouse orthologs (IC_{50} = 25±3.9 µM; Fig. 3c). Like for the other $P2Y_4$ receptors this inhibition was not competitive (Fig. 4c).

3.3. Tissue distribution of the mouse $P2Y_4$ transcript

The tissue distribution of the $P2Y_4$ receptor was investigated by RT-PCR, using specific primers, on total RNA extracted from 16 different mouse tissues (brain, heart, lung, thymus, spleen, kidney, liver, pancreas, muscle, intestine, stomach, salivary glands, bladder, testis, ovary and uterus). As shown in Fig. 5, the mouse $P2Y_4$ transcript was strongly amplified from liver, intestine and stomach extracts. It was also detected in lung and bladder but with a lower intensity. The band observed with the heart is non-specific since its size is at least 100 bp above that of the 498 bp product expected.

4. Discussion

We have isolated and cloned the mouse ortholog of the P2Y$_4$ receptor. The predicted amino acid sequences of the human, rat and mouse P2Y$_4$ receptors are highly conserved (Fig. 1). The agonist profile for the mouse P2Y$_4$ expressed in the 1321N1 cell line matches the profile described for the rat P2Y$_4$ receptor. Both are activated by UTP and ATP with almost the same potency (EC$_{50}$ around 1 μM) (Bogdanov et al., 1998, Webb et al., 1998). In the P2Y$_2$ receptor, which is also activated by UTP and ATP, three conserved amino acid residues (H^{262}, R^{265} and R^{296}) have been shown to be crucial for the binding of both agonists (Erb et al., 1995). These amino acids are also conserved in the three P2Y$_4$ orthologs (Fig. 1) although ATP is only a partial agonist or an antagonist for the human P2Y$_4$ (Communi et al., 1995; Kennedy et al., 2000). The antagonist profile of the mouse P2Y$_4$ receptor is similar to the one described for the human P2Y$_4$. Indeed, PPADS was the most active antagonist of this receptor with an IC$_{50}$ value around 50 μM, followed by Reactive Blue 2, while suramin was inactive. This rank of activity is similar to the one determined for the human P2Y$_4$ (Communi et al., 1996). In another study, PPADS was found to be only a weak antagonist of the human P2Y$_4$ receptor (Charlton et al., 1996). These authors even described an increase of UTP stimulation by PPADS at lower concentration. The experimental conditions used by these authors were not exactly the same, but no precise explanation of the discrepancy can be provided. Bogdanov et al. (1998) reported, without actually showing the data, that PPADS had no activity on the rat P2Y$_4$ receptor expressed in *Xenopus* oocytes. We show here that when the rat P2Y$_4$ receptor is expressed in 1321N1 cells, PPADS is able to inhibit the UTP-stimulation of this receptor. The exact reason for the discrepancy is unclear, but might obviously be related to the different expression system used. Like for the human and the rat orthologs, Reactive Blue 2 is also a potent inhibitor of the mouse P2Y$_4$, with

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an IC_{50} value similar to the one measured for PPADS. Like for the human $P2Y_4$, suramin was inactive on the mouse ortholog. We have also observed that when the rat P2Y4 is expressed in 1321N1 cells, suramin at 100 µM was unable to inhibit the UTP-responses (data not shown), while in *Xenopus* oocytes suramin weakly antagonized UTP-responses (Bogdanov et al., 1998). A direct effect of suramin on the particular G protein coupled to $P2Y_4$ receptor in *Xenopus* oocytes cannot be excluded. In conclusion, when compared in the same experimental model, the mouse and rat $P2Y_4$ are equivalent in terms of agonists and antagonists profile.

Concerning the tissue distribution of the $P2Y_4$, little is known about the human ortholog, the messenger RNA of which has been detected in the placenta, peripheral blood leukocytes and several human lung cell lines (Communi et al., 1995; Jin et al., 1998; Communi et al., 1999). The rat $P2Y_4$ receptor messenger distribution has been characterized by RT-PCR analysis (Webb et al., 1998). The transcript was detected in brain, spinal cord, heart and a variety of other peripheral organs. In this study, we detected, by RT-PCR, a strong signal corresponding to the mouse $P2Y_4$ messenger in extracts of liver, stomach and intestine. These last observations are in accordance with recent data suggesting that the UTP and ATP Cl^- secretory response could be mediated by the $P2Y_4$ receptor in the mouse jejunum (Cressman et al., 1999). The mouse $P2Y_4$ messenger has also been detected in the lung tissue, like the human ortholog (Communi et al., 1999). This raises the possibility that the $P2Y_4$ receptor could mediate the residual effect of ATP and UTP on the Cl^- transport by the tracheal epithelium of $P2Y_2$ -/- mice (Cressman et al., 1999). Contrarily to what is observed for the rat receptor, the mouse $P2Y_4$ messenger was not detected in the brain or in the heart (Webb et al., 1998).

In conclusion, in terms of agonist stimulation, the mouse P2Y$_4$ is activated by UTP and ATP and is closer to the rat ortholog, while in terms of antagonist profile, the three P2Y$_4$ receptors are similar.

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Fig. 1 Alignment of the three cloned P2Y4 sequences. The amino acid sequences of rar P2Y$_4$ (361aa; Y14705), mouse P2Y$_4$ (361aa; AJ277752) and human P2Y$_4$ (365aa; X91852) are aligned. Hydrophobic regions (transmembrane domains) are indicated by bars. Identical amino acids are indicated by (*), conserved amino acids by (:). Gaps (-) were introduced to maximize the alignment.

Fig. 2 Concentration-action curves of UTP and ATP on the InsP$_3$ accumulation in mouse P2Y$_4$ receptor-transfected 1321N1 cells. The cells were incubated 30 sec in the presence of various UTP and ATP concentrations (0.03, 0.1, 0.3, 1, 3, 30 and 100 µM). The data represent the mean±s.e.mean from three independant experiments each in triplicate. The EC$_{50}$ values are: EC$_{50}$ UTP = 0.4±0.08 µM and EC$_{50}$ ATP = 0.7±0.3 µM (mean±S.D. of EC$_{50}$ values obtained from three independent experiments).

Fig. 3 Concentration-dependence of PPADS and Reactive Blue 2 inhibition of the UTP-response in mouse (a, b) and rat $P2Y_4$ (c) receptor-transfected 1321N1 cells. The cells were exposed to various concentrations (1, 3, 10, 30, 100, 300 μM and 1 mM) of PPADS (a, c) and Reactive Blue 2 (b) for 20 min. UTP at a concentration of 1 μM was then added for 30 sec. For the mouse $P2Y_4$ receptor, the IC_{50} PPADS = 45±15 μM and IC_{50} Reactive Blue 2 = 47±12.5 μM. For the rat $P2Y_4$ receptor, the IC_{50} PPADS = 25±3.9 μM. In a and b, data are means±s.e.mean from 3 separate experiments each in triplicate. In c, data are means±range from 2 separate experiments each in triplicate.

Fig. 4 Effect f PPADS and Reactive Blue 2 on the UTP stimulation of InsP$_3$ in 1321N1-transfected cells. The mouse P2Y$_4$-transfected cells (a and b) and rat P2Y$_4$-transfected cells (c) were incubated for 20 min in the presence or the absence of two concentrations (50 and 300 μM) of PPADS (a, c) or Reactive Blue 2 (b), and then exposed to various concentrations of UTP for 30 sec. The data represent the mean±range from two separate experiments each in triplicate.

Fig. 5 Detection of mouse P2Y4 mRNA in mouse tissues by RT-PCR experiments. The extraction of RNA and the reverse transcription were performed as described under "Materials and methods". PCR products of 498 bp are visualized after electrophoresis on a 1.5% agarose gel and ethidium bromide coloration. Only 7 of the 16 tissues tested are shown.

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mP2Y₄   MTSADSLLFTSLGPSPSSGDG----DCKFNEEFKFILLP
rP2Y₄   MTSAESLLFTSLGPSPSSGDG----DCRFNEEFKFILLP
hP2Y₄   MASTESSLLRSLGLSPGPGSSEVELDCWFDEDFKFILLP
        *:*::* *: *** ** *      ** *:*:*******

mP2Y₄   LSYAVVFVLGLALNAPTLWLFLFRLRPWDATATYMFHLA
rP2Y₄   MSYAVVFVLGLALNAPTLWLFLFRLRPWDATATYMFHLA
hP2Y₄   VSYAVVFVLGLGLNAPTLWLFIFRLRPWDATATYMFHLA
        :*********** ********:*****************

mP2Y₄   LSDTLYVLSLPTLVYYYAARNHWPFGTGFCKFVRFLFYW
rP2Y₄   LSDTLYVLSLPTLVYYYAARNHWPFGTGLCKFVRFLFYW
hP2Y₄   LSDTLYVLSLPTLIYYYAAHNHWPFGTEICKFVRFLFYW
        ************:*****:*******  :**********

mP2Y₄   NLYCSVLFLTCISVHRYMGICHPLRAIRWGRPRFAGLLC
rP2Y₄   NLYCSVLFLTCISVHRYLGICHPLRAIRWGRPRFASLLC
hP2Y₄   NLYCSVLFLTCISVHRYLGICHPLRALRWGRPRLAGLLC
        ****************:*********:******:* ***

mP2Y₄   LGVWLVVAGCLVPNLFFVTTNANGTTILCHDTTLPEEFD
rP2Y₄   LGVWLVVAGCLVPNLFFVTTNANGTTILCHDTTLPEEFD
hP2Y₄   LAVWLVVAGCLVPNLFFVTTSNKGTTVLCHDTTRPEEFD
        *  ****************    :***:****** ****

mP2Y₄   HYVYFSSTIMVLLFGFPFLITLVCYGLMARRLYRPLPGA
rP2Y₄   HYVYFSSAVMVLLFGLPFLITLVCYGLMARRLYRPLPGA
hP2Y₄   HYVHFSSAVMGLLFGVPCLVTLVCYGLMARRLYQPLPGS
        ***:***::* **** * *:*************:****:

mP2Y₄   GQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYLARL
rP2Y₄   GQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYQARL
hP2Y₄   AQSSSRLRSLRTIAVVLTVFAVCFVPFHITRTIYYLARL
        ***********************************  ***

mP2Y₄   LNAECRVLNIVNVVYKVTRPLASANSCLDPVLYLFTGDK
rP2Y₄   LQADCHVLNIVNVVYKVTRPLASANSCLDPVLYLFTGDK
hP2Y₄   LEADCRVLNIVNVVYKVTRPLASANSCLDPVLYLLTGDK
        *:*:*:*********************************:****

mP2Y₄   YRNQLQQLCRGSTPKRRTTASSLALVTLHEESISRWADI
rP2Y₄   YRNQLQQLCRGSKPKPRTAASSLALVTLHEESISRWADT
hP2Y₄   YRRQLRQLCGGGKPQPRTAASSLALVSLPEDSSCRWAAT
        ** **:*** *  *: **:*******:*  *:*   ***

mP2Y₄   HQDSIFPAYEGDRL  361
rP2Y₄   HQDSTFSAYEGDRL  361
hP2Y₄   PQDSSCSTPRADRL  365
        ***      :   ***
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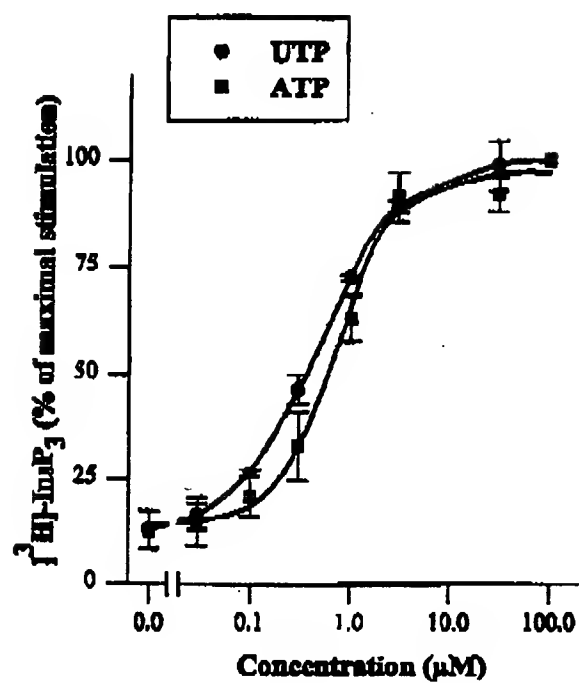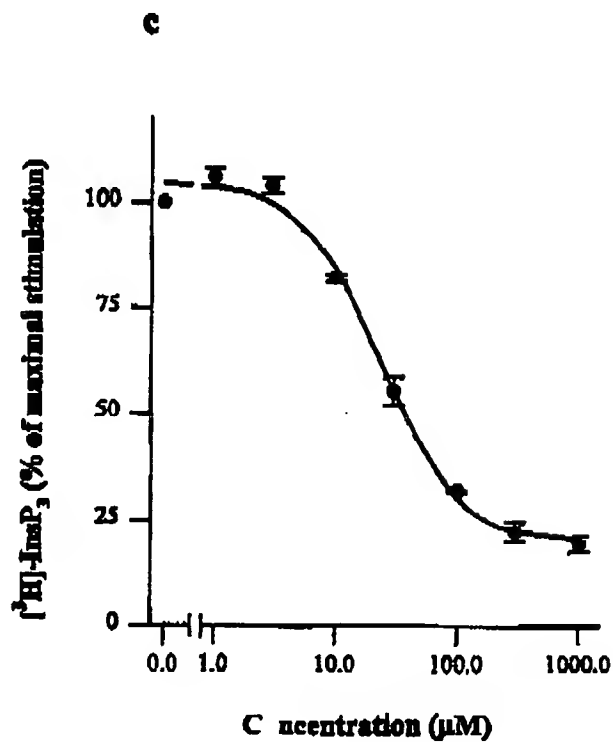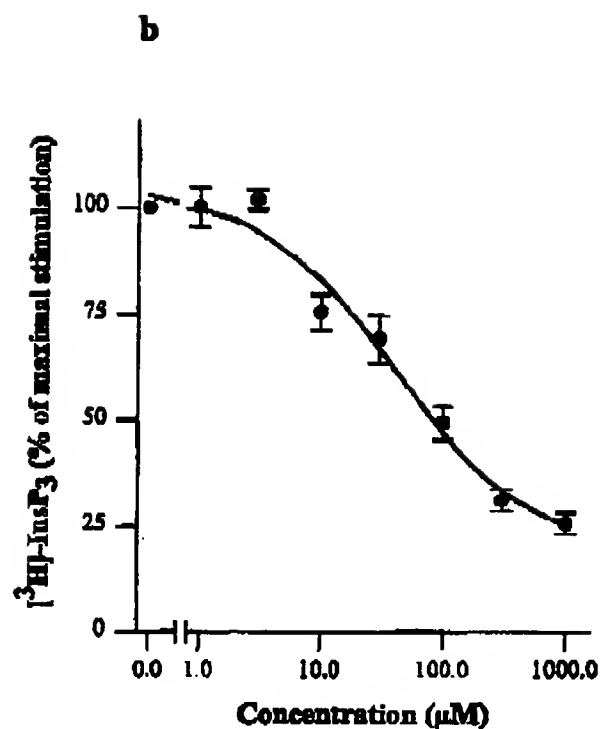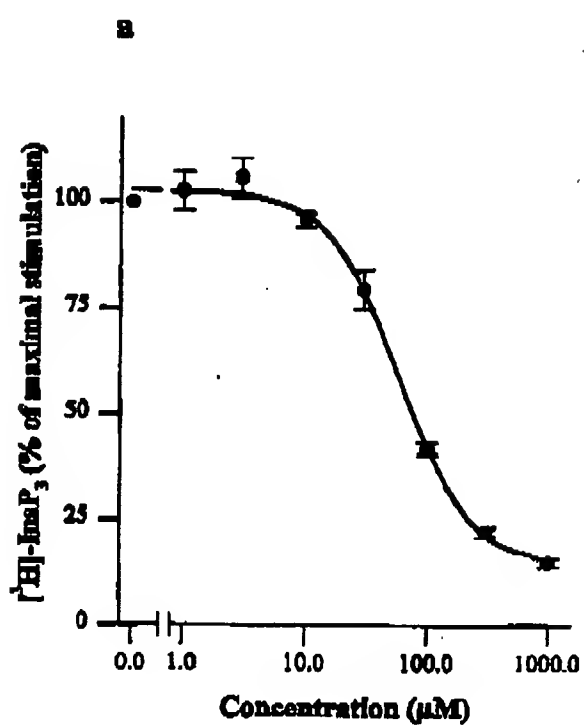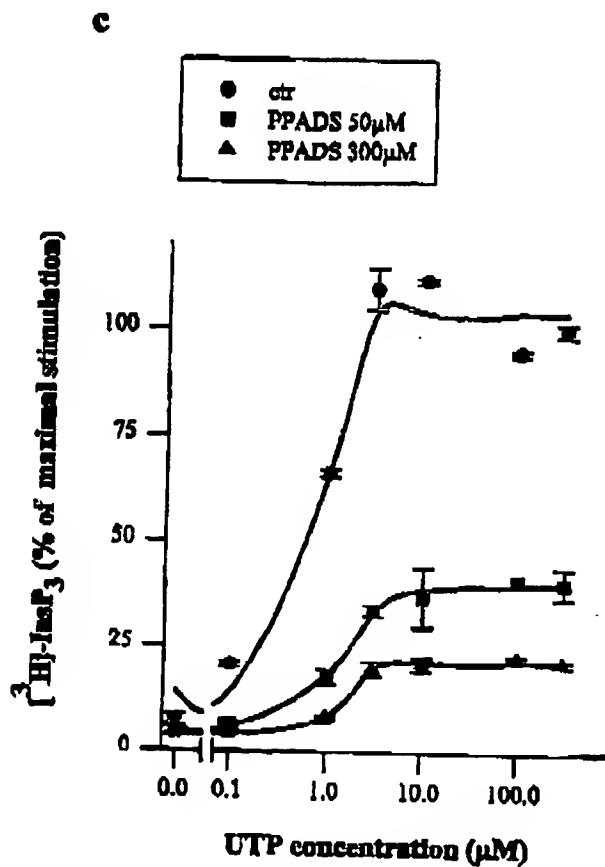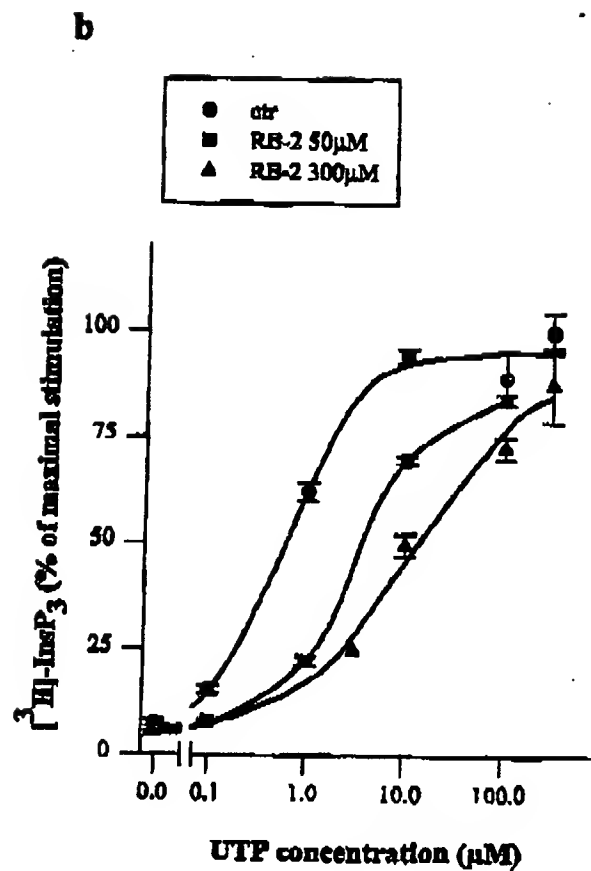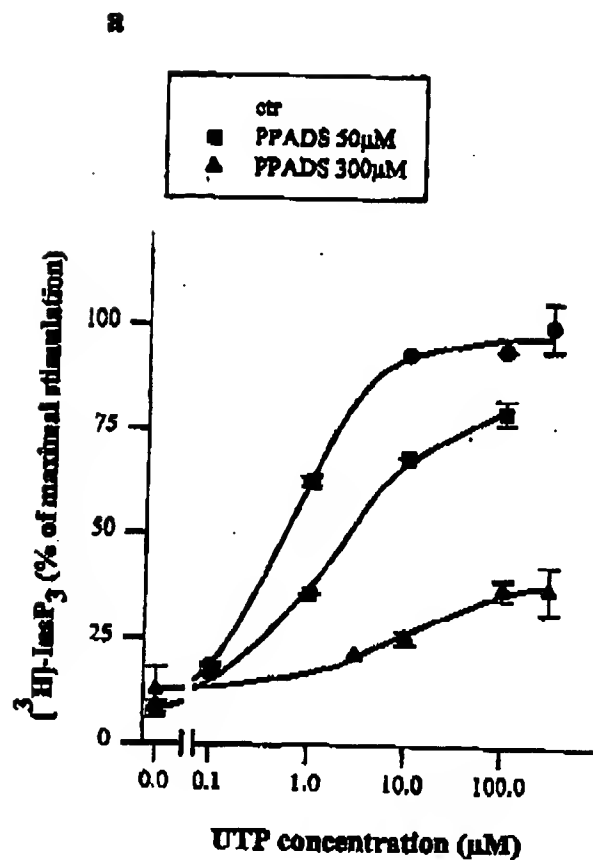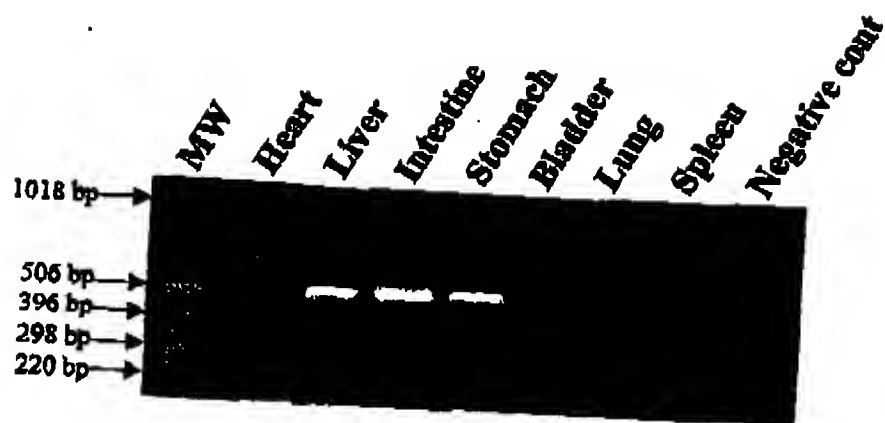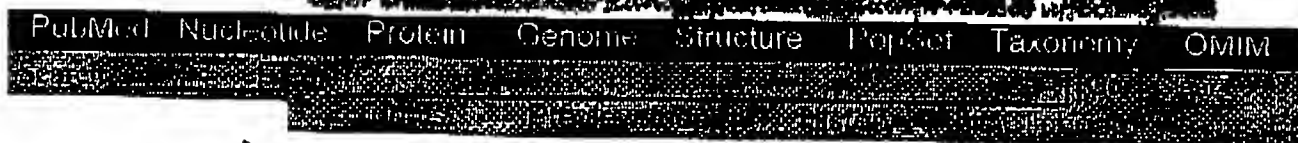
Fig. 1

Fig. 2

a

b

c

Fig. 2

a

b

c

Fig. 5

EXHIBIT B

Entrez PubMed

Molecular cloning and characterization of the rat P2Y4 receptor.

Webb TE, Henderson DJ, Roberts JA, Barnard EA

Molecular Neurobiology Unit, Royal Free Hospital School of Medicine, London, England.

Degenerate PCR was used to amplify DNAs encoding members of the P2Y receptor family from rat brain RNA. A full-length sequence obtained for one novel clone (R5) contained an intronless open reading frame that encoded a polypeptide of 361 amino acids, sharing 84% sequence identity with the human P2Y4 receptor. When R5 was stably expressed in Jurkat cells, calcium fluxes resulting from stimulation of the receptor showed that UDP, ADP, 2-methylthio-ATP, and diadenosine tetraphosphate were inactive, whereas UTP and ATP were both full agonists with similar potency. At the human receptor, ATP has significantly lower potency than UTP. The R5 transcript was not detected in brain by northern hybridization. Therefore, its tissue distribution was assessed by PCR, and the mRNA was found to be widely distributed at a low abundance, being present in brain, spinal cord, and a variety of peripheral organs. Localization of the receptor transcript in adult rat brain sections by in situ hybridization indicated that it is expressed at highest levels in the pineal gland and ventricular system. It is presumed that R5 is a species orthologue of the human P2Y4 receptor but with this significant difference in agonist pharmacology.

PMID: 9751165, UI: 98421785

EXHIBIT C
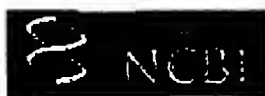
☐1: *Br J Pharmacol* 1998 Jun;124(3):428-30   Books, LinkOut

Molecular cloning and characterization of rat P2Y4 nucleotide receptor.

Bogdanov YD, Wildman SS, Clements MP, King BF, Burnstock G

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An intronless open reading frame encoding a protein (361aa in length) was isolated from a rat genomic library probed with a DNA fragment from rat heart. This protein showed 83% sequence identity with the human P2Y4 (hP2Y4) receptor and represents a homologue of the human pyrimidinoceptor. However, the rP2Y4 receptor is not selective for uridine nucleotides and, instead, shows an agonist potency order of ITP = ATP = ADP(pure) = UTP = ATPgammaS = 2-MeSATP = Ap4A > UDP(pure). ADP, ATPgammaS, 2-MeSATP and UDP are partial agonists. Thus, in terms of agonist profile, rP2Y4 is more like the P2U receptor subtype. The rP2Y4 receptor was reversibly antagonized by Reactive blue 2 but not by suramin which, otherwise, inhibits the hP2Y2 receptor (a known P2U receptor). Thus, rP2Y4 and the P2Y2 subtype appear to be structurally distinct forms of the P2U receptor (where ATP and UTP are equi-active) but can be distinguished as suramin-insensitive and suramin-sensitive P2U receptors, respectively.

Cloning and expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy

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ABSTRACT The Cl^- secretory pathway that is defective in cystic fibrosis (CF) can be bypassed by an alternative pathway for Cl^- transport that is activated by extracellular nucleotides. Accordingly, the P_2 receptor that mediates this effect is a therapeutic target for improving Cl^- secretion in CF patients. In this paper, we report the sequence and functional expression of a cDNA cloned from human airway epithelial (CF/T43) cells that encodes a protein with properties of a P_{2U} nucleotide receptor. With a retrovirus system, the human airway clone was stably expressed in 1321N1 astrocytoma cells, a human cell line unresponsive to extracellular nucleotides. Studies of inositol phosphate accumulation and intracellular Ca^{2+} mobilization induced by extracellular nucleotides in 1321N1 cells expressing the receptor identified this clone as the target receptor in human airway epithelia. In addition, we independently isolated an identical cDNA from human colonic epithelial (HT-29) cells, indicating that this is the same P_{2U} receptor that has been functionally identified in other human tissues. Expression of the human P_{2U} receptor (HP2U) in 1321N1 cells revealed evidence for autocrine ATP release and stimulation of transduced receptors. Thus, HP2U expression in the 1321N1 cell line will be useful for studying autocrine regulatory mechanisms and in screening of potential therapeutic drugs.

The transepithelial movement of fluid is coupled to the transport of Cl^- and other electrolytes, and in airway tissue the regulation of this activity is vital to normal function. Defective airway epithelial Cl^- secretion is a principal characteristic of cystic fibrosis (CF) (1). This defect contributes to the development of dehydrated mucus, which obstructs airways and compromises lung function. Acting as signaling molecules, extracellular 5′-nucleotides elicit diverse responses in a variety of tissues (2–4). In airway epithelia, ATP and UTP activate an alternative, non-CF transmembrane conductance regulator (CFTR)-dependent Cl^- conductance (5), raising the possibility that nucleotides may be used therapeutically to induce Cl^- secretion in the airways of individuals with CF (6, 7). Extracellular ATP and UTP also stimulate mucus secretion by goblet cells (8, 9) *in vitro*, and excessive activation of this pathway *in vivo* may contribute to hypersecretion in chronic bronchitis. Isolation and molecular characterization of the receptor for extracellular nucleotides present in human airway and other epithelia will permit studies on the expression of this receptor in normal and diseased tissues and facilitate identification of drugs for therapy.

Recently, a murine P_{2U} receptor cDNA was cloned from neuroblastoma–glioma hybrid (NG108-15) cells (10) which, when expressed in K562 human leukemia cells (11), encodes a 53-kDa protein that exhibits pharmacological and signaling

properties similar to a P_{2U} nucleotide receptor that regulates ion transport in human airway (6, 12) and intestinal (13) epithelia. We used the murine P_{2U} receptor amino acid sequence to isolate human P_{2U} receptor cDNAs from CF/T43 (human airway) and HT-29 (human colonic) epithelial cell libraries. The human cDNAs are identical[§] and were functionally characterized by expression in human cells that lack endogenous P_{2U} receptor responses. The 1321N1 astrocytoma cell expression system, in addition to being useful for the pharmacological characterization of retrovirally transduced cDNA, constitutes a possible model for the previously postulated autocrine P_2 receptor regulation of cell function (4).

EXPERIMENTAL PROCEDURES

cDNA Cloning and Sequencing. Degenerate oligonucleotide primers [5′-AATGG(C/A/G)AC(C/T/A)TGGGA(G/A)-GG(G/A)GA(C/T)GA(A/G)-3′ and 5′-GACGTG(C/G)-AA(A/G)GGCAG(A/C)(A/C)AGC(A/T)GAGGGCGA-A-3′] based on the murine P_{2U} receptor sequence (10) were used in low-stringency PCR to amplify products from a cDNA library constructed in λUni-ZAP XR (Stratagene) from CF/T43 cell poly(A)$^+$ RNA. Products were cloned into pCR II (Invitrogen) and screened by Southern blot using probe P263, a random primer-labeled partial cDNA (corresponding to amino acids 8–276) generated by high-stringency PCR amplification of the murine P_{2U} receptor clone.

A cloned PCR product of about 500 bases (probe D9) that hybridized with probe P263 was labeled by random priming and used to screen the CF/T43 cDNA library. Hybridization conditions were as described below for Northern and Southern blots. Phage from one plaque, which hybridized strongly with probe D9, was purified by additional screening and pBluescript SK(−) (Stratagene) was rescued by *in vivo* excision. In parallel, an HT-29 cDNA library prepared in λgt10 was screened with probe P263, and insert from a positive plaque-purified phage was subcloned into the *Not* I site of pBluescript. Plasmid DNAs were purified by CsCl gradient centrifugation (14), and both strands of the two clones were sequenced by dideoxy chain termination (Sequenase version 2.0; United States Biochemical).

Heterologous Expression. A retroviral vector-containing plasmid, pLHP2USN, was constructed by insertion of the cloned CF/T43 cDNA into the *Eco*RI and *Xho* I sites of pLXSN (15). An amphotrophic packaging cell line, PA317, was used to produce the LHP2USN retroviral vector and a control vector containing only the neomycin-resistance

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Abbreviations: CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; $[Ca^{2+}]_i$, intracellular concentration of free Ca^{2+}; HP2U, human P_{2U} receptor.
[§]The sequence reported in this paper has been deposited in the GenBank database (accession no. V07225).

(*neo^r*) gene (LN) (15). Human astrocytoma cells (1321N1) generously provided by T. K. Harden (University of North Carolina, Chapel Hill) were infected with LHP2USN r LN (2 hr with Polybrene at 4 μg/ml) and, after 48 hr, selected with G418 (600 μg/ml; Life Technologies, Grand Island, NY). To assay f r receptor activity, the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was measured in confluent cells on coverslips by fura-2 fluorescence with microspectrofluorimetry (16). Measurement of cell inositol phosphate formation was as described (12). The cloned HT-29 cDNA was transiently expressed and characterized in K562 cells as described (11).

Northern and Southern Blots. Total cell RNA and poly(A)$^+$ RNA were isolated by standard procedures or purchased commercially (Clontech). Human and mouse genomic DNAs were extracted from cultured cells (14) and digested with restriction enzyme (5 units/μg of DNA). Northern and Southern blots were prepared (14) and UV-crosslinked (Stratagene). Prehybridization and hybridization with cDNA probes were performed using QuikHyb (Stratagene) according to the manufacturer's instructions, except that 0.2× standard saline citrate (SSC)/0.1% SDS was used for the high-stringency wash. Autoradiographs were prepared with Kodak XAR film and one intensifying screen at −80°C.

RESULTS AND DISCUSSION

Full-length clones obtained by screening human CF/T43 and HT-29 cDNA libraries were found by sequence analysis to contain identical cDNA inserts. The amino acid sequence deduced from an 1128-bp open reading frame (Fig. 1) bears substantial similarity (89% identity) to the mouse P_{2U} receptor sequence reported by Lustig *et al.* (10), with the majority of the sequence differences concentrated in the carboxyl-terminal region. The HP2U sequence is considerably less similar to the chicken P_{2Y} purinoceptor (37% identity) described by Webb *et al.* (17). The HP2U sequence exhibits structural features typical of the family of G protein-coupled receptors and common to both the chicken and murine P_2 receptor clones: (*i*) seven hydrophobic domains, (*ii*) consensus N-linked glycosylation sequences near the amino terminus, (*iii*) a number of residues highly conserved among G protein-coupled receptors (e.g., Asn^{51}, Asp^{79}, Cys^{106}, and Cys^{183}), and (*iv*) potential phosphorylation sites in the third intracellular and carboxyl-terminal domains. Like the mouse and chicken P_2 receptors, the HP2U sequence is more closely related to the cloned G protein-coupled receptors for peptide h rmones than to those for adenosine and cAMP.

A retroviral system was used to obtain stable expression of the CF/T43 HP2U clone in 1321N1 cells, a human astrocytoma cell line that is unresponsive to classical P_2 receptor agonists. The effect of extracellular nucleotides on $[Ca^{2+}]_i$ was assessed in CF/T43 cells, uninfected 1321N1 cells, and 1321N1 cells infected with retroviral vector containing either the airway HP2U cDNA (LHP2USN) or no insert (LN). Exposure of CF/T43 cells to extracellular UTP (0.1 mM) in Ca^{2+}-containing medium resulted in an initial rapid increase in $[Ca^{2+}]_i$ which relaxed to a plateau (Fig. 2A). Application of UTP to CF/T43 cells in Ca^{2+}-free medium induced a rapid increase in $[Ca^{2+}]_i$ which returned to baseline without a plateau. Extracellular UTP had no effect on $[Ca^{2+}]_i$ in uninfected 1321N1 cells (data not shown) or LN-infected cells, whereas carbachol elicited large responses. The $[Ca^{2+}]_i$ response to UTP in 1321N1 cells xpressing the LHP2USN vector was similar to that of CF/T43 cells in both Ca^{2+}-free and Ca^{2+}-containing media. In each experiment, the addition of ATP produced the same result observed with UTP (data not shown).

T examine the pharmacological specificity of the $[Ca^{2+}]_i$ response in 1321N1 cells expressing the HP2U clone, concentration–effect curves were generated for UTP, ATP, and

FIG. 1. Human P_{2U} receptor (HP2U) nucleotide and deduced amino acid sequence. The putative transmembrane domains are underlined.

structural analogues of ATP selective for P_{2X} (adenosine 5'-[α,β-methylene]triphosphate) and P_{2Y} (2-methylthio-ATP) receptors (Fig. 2B). UTP and ATP were nearly equipotent, with UTP exhibiting slightly more efficacy, while 2-methylthio-ATP and adenosine 5'-[α,β-methylene]triphosphate had little effect. Similar concentration–effect curves were generated for UTP and ATP in K562 cells, a cell line that also lacks endogenous P_{2U} receptors (11), transiently transfected with HP2U (data not shown). Clearly, the agonist specificities most closely fit the pharmacological classification of the P_{2U} receptor (18).

Pretreatment of CF/T43 and HP2U-1321N1 cells with pertussis toxin inhibited ATP-stimulated intracellular Ca^{2+} mobilization in both cell types by 20–30% (Fig. 2C). These results are consistent with the partial inhibition by pertussis toxin on inositol phosphate accumulation induced by ATP or UTP in CF/T43 cells (12) and on P_{2U} receptor responses in other systems (11, 19). The pertussis t xin sensitivity of HP2U-1321N1 cells and the observation that UTP or ATP increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} are consistent with the predicted coupling of HP2U to ph spholipase C via a G protein. T test this possibility more directly, we measured inosit l ph sphate formation.

P_{2U} receptor-activated Ca^{2+} responses are linked to activation of inositolph spholipid hydrolysis in various cells (4,

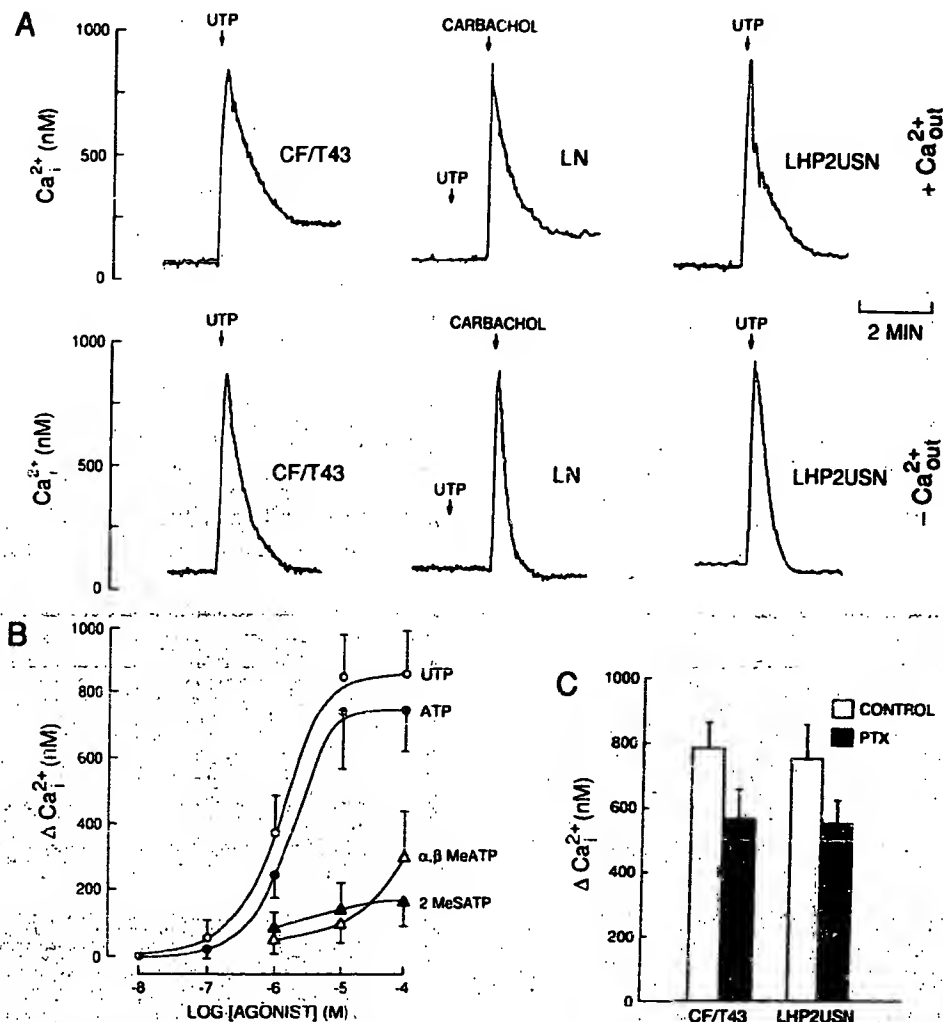Medical Sciences: Parr et al.

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FIG. 2.   (A) $[Ca^{2+}]_i$ response to UTP (0.1 mM) and/or carbachol (0.1 mM) in CF/T43 cells and in LN-infected and LHP2USN-infected 1321N1 astrocytoma (LN- and LHP2USN-1321N1, respectively) cells bathed in Ca^{2+}-containing (1.3 mM Ca^{2+}; upper row) or Ca^{2+}-free (1.0 mM EGTA; lower row) NaCl/Ringer solutions. In the presence of extracellular Ca^{2+}, basal $[Ca^{2+}]_i$ averaged 72 ± 8 nM (mean ± SEM, n = 32), 67 ± 11 nM (n = 30), and 68 ± 5 nM (n = 123) in CF/T43, LN-1321N1, and LHP2USN-1321N1 cells, respectively. In Ca^{2+}-free solution, the mean basal $[Ca^{2+}]_i$ values were 65 ± 7 nM (n = 18), 64 ± 7 nM (n = 23), and 63 ± 6 nM (n = 22) in CF/T43, LN-1321N1, and LHP2USN-1321N1 cells, respectively. There was no significant difference in basal $[Ca^{2+}]_i$ (P > 0.05; Student's t test) between the different cell preparations or between cells bathed with Ca^{2+}-containing medium and cells bathed with Ca^{2+}-free medium. In the presence of extracellular Ca^{2+} the mean change in $[Ca^{2+}]_i$ (peak − basal value) in response to UTP was 823 ± 145 nM (n = 7) and 881 ± 119 nM (n = 10) in CF/T43 and LHP2USN-1321N1 cells, respectively. In Ca^{2+}-free Ringer solution, the mean change in $[Ca^{2+}]_i$ was 807 ± 123 nM (n = 7) and 867 ± 112 nM (n = 8) in CF/T43 and LHP2USN-1321N1 cells, respectively. There was no significant difference in the mean change in $[Ca^{2+}]_i$ in response to UTP between CF/T43 cells and LHP2USN-1321N1 cells (P > 0.05) in either the presence or absence of extracellular Ca^{2+}. In the presence and absence of extracellular Ca^{2+}, carbachol induced mean changes in $[Ca^{2+}]_i$ of 863 ± 132 nM (n = 10) and 834 ± 141 nM (n = 8), respectively, in LN-1321N1 cells. These values do not differ significantly (P > 0.05) from the UTP-elicited $[Ca^{2+}]_i$ changes in CF/T43 and LHP2USN-1321N1 cells. (B) Concentration-effect relationships of different purine and pyrimidine compounds on changes in $[Ca^{2+}]_i$ (ΔCa^{2+}_i; peak − basal values) in LHP2USN-infected 1321N1 cells bathed with Ca^{2+}-containing NaCl/Ringer solution. Each point is the mean ± SEM for five or more separate experiments. α,β MeATP, adenosine 5'-[α,β-methylene]triphosphate; 2MeSATP, 2-methylthio-ATP. (C) Effects of pertussis toxin (PTX; 10 ng/ml for 24 hr) on ATP (0.1 mM)-induced changes in $[Ca^{2+}]_i$ (ΔCa^{2+}_i) in CF/T43 and LHP2USN-1321N1 cells bathed with Ca^{2+}-containing NaCl/Ringer solution. For control and PTX-treated CF/T43 cells, the average change in $[Ca^{2+}]_i$ was 788 ± 84 nM (n = 14) and 568 ± 88 nM (n = 14), respectively. For control and PTX-treated LHP2USN-infected 1321N1 cells, the average change in $[Ca^{2+}]_i$ was 765 ± 116 nM (n = 12) and 558 ± 71 nM (n = 8), respectively. For both PTX-treated CF/T43 and LHP2USN-1321N1 cells, the average change in $[Ca^{2+}]_i$ was significantly lower (P < 0.05) than in control cells.

20, 21), and inositol phosphate formation was detected in HP2U-1321N1 cells incubated with UTP and ATP (Fig. 3). In our initial studies, very high levels of inositol phosphates were found to accumulate in HP2U-1321N1 cells that had not been exposed t exogenously added nucleotides (Fig. 3A). One possible explanation for the higher basal levels—i.e., intrinsic, agonist-independent (constitutive) receptor activation—is not supported by the large, reproducible $[Ca^{2+}]_i$ modulations observed in response t nucleotide additi n

(Fig. 2). Therefore, we hypothesized that during the lengthy labeling period the 1321N1 cells released 5'-nucleotides into the medium in quantities sufficient to "self-activate" the expressed receptor. To test this notion, the phosphatase apyrase was added to the medium during cell labeling to metabolize extracellular ATP. The inclusion of apyrase resulted in a reduction of baseline inositol phosphate levels to values near those of controls (Fig. 3A). Further, preliminary HPLC analysis (22) of medium bathing HP2U-1321N1 cells
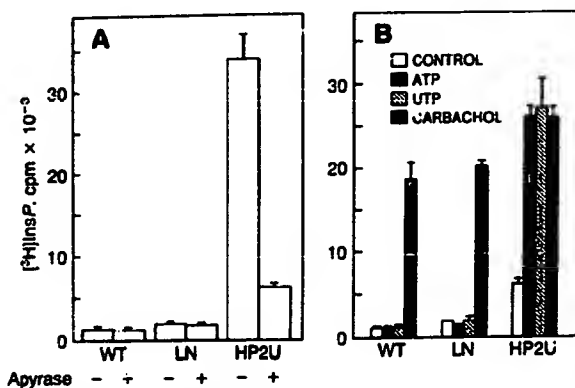
FIG. 3.   (A) [³H]Inositol phosphate ([³H]InsP) formation in uninfected (wild type, WT), LN-infected (LN), and LHP2USN-infected (HP2U) 1321N1 astrocytoma cells. Cells were labeled for 18 hr with [³H]inositol in the absence (−) or presence (+) of apyrase (2 units/ml). After removal of unincorporated radioactivity, the cells were incubated for 15 min with 10 mM LiCl and the total inositol phosphates accumulated in the absence of added agonists were measured. (B) [³H]Inositol phosphate formation in response to UTP (0.1 mM), ATP (0.1 mM), or carbachol (1 mM) in uninfected (WT), LN-infected (LN), and LHP2USN-infected (HP2U) 1321N1 astrocytoma cells. Cells were labeled in the presence of apyrase (2 units/ml), preincubated with LiCl as above, and then challenged for 15 min with or without the indicated agonists. Data represent the mean ± SD of triplicate determinations and are representative of results obtained in two separate experiments. The results were normalized with the total radioactivity (150,000–300,000 cpm) present in the lipid fraction.

prelabeled with [³H]adenine showed that 1.5% of the intracellular [³H]ATP pool (total intracellular ATP, 140 pmol per 10⁶ cells) accumulated in the extracellular compartment as [³H]ATP. Thus, it appears that astrocytoma cells expressing HP2U are persistently stimulated by nucleotides in an autocrine fashion. The mode of release of nucleotides from astrocytoma cells is unknown; however, noncytolytic mechanisms of ATP release have been described or proposed for a variety of neuronal, secretory, and other cell types (reviewed in ref. 4). Since many of the cell types that release ATP also endogenously express nucleotide receptors, expression of the P_{2U} receptor in 1321N1 cells may have rendered this physiologically important regulatory mechanism accessible to further study.

The addition of apyrase to the medium facilitated comparison of agonist-induced inositol phosphate accumulation in control and LHP2USN-infected cells. Accordingly, experiments for the characterization of HP2U were performed on cells pretreated with apyrase during the labeling procedure. Consistent with activation by HP2U of phospholipase C, ATP and UTP increased inositol phosphate accumulation in 1321N1 cells expressing LHP2USN but not in uninfected or LN-infected controls (Fig. 3B).

P_{2U} receptors have been functionally described in various human organs and cell types (4). As shown in Fig. 4A, P_{2U} receptor mRNA is widely distributed in human tissue, including the heart, liver, lung, and kidney, as reported for the mouse (10), and placenta and skeletal muscle. Consistent with studies showing P_{2U} receptor-mediated modulation of epithelial ion transport, mRNA was detected in kidney proximal-tubule cells and the salivary gland duct cell line HSG-PA (data not shown) and in primary cultures of nasal epithelium, a tissue representative of the therapeutic site in airways for P_{2U} receptor regulation of ion transport (6, 7).

Some human tissues, including nasal and proximal-tubule epithelia and liver, express only a 2.1-kb mRNA; however, as many as three mRNAs were observed (additional bands at 7.5 kb and/or 9 kb) in other human tissues and cell lines,
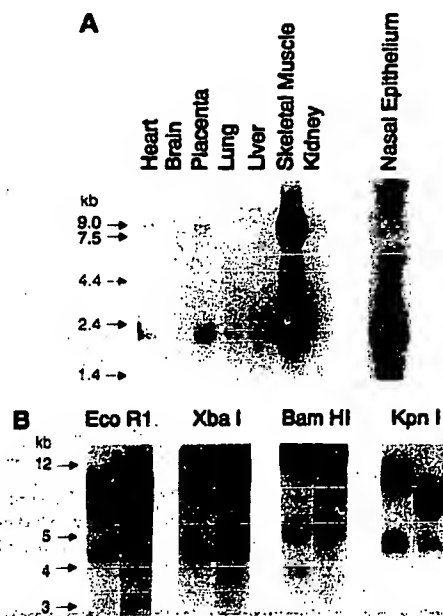

FIG. 4.   (A) Northern blot analysis of RNAs isolated from various human tissues. Total RNA (20 µg; primary cultures of nasal epithelium) and poly(A)⁺ RNA (2 µg) were subjected to hybridization analysis using random primer-labeled full-length HP2U cDNA (nasal epithelium) or the coding-sequence cDNA fragment D9 as probes. (B) Southern blot analysis of genomic DNA. Human genomic DNA (10 µg) was digested with the indicated restriction enzymes and subjected to hybridization analysis using random primer-labeled HP2U coding-sequence cDNA fragment D9 (left lanes) or a Kpn I fragment excised from the HP2U clone (right lanes) as probes.

including CF/T43 and HT-29 cells (data not shown). Although all of the bands in human tissues were found to cross-hybridize with a full-length murine P_{2U} receptor cDNA, none of the murine tissues analyzed (10) contain more than one transcript. Whereas it is possible that the larger transcripts in human tissues and cells represent unprocessed forms of the 2.1-kb mRNA, the relative abundance of the hybridizing bands suggests that the larger RNAs are as stable as the smaller RNA and, therefore, may not represent an intermediate step in processing. Further, significant amounts of the larger RNAs were retained on an oligo(dT)-cellulose column, indicating that these mRNAs are polyadenylylated. If all of the bands represent fully processed mRNAs, they may represent alternatively processed forms of the same gene or products of different genes. Southern blot analysis of human genomic DNA was performed to explore the possibility that more than one gene for P_{2U} receptors exist in the human genome.

Human genomic DNA was digested to completion with the restriction enzymes EcoRI, Xba I, BamHI, and Kpn I. Among these, only Kpn I will cut in the HP2U cDNA. Under stringent conditions, the full-length HP2U cDNA hybridized to two or more fragments in all genomic DNA digests (data not shown). Since the multiple bands in the EcoRI, Xba I, and BamHI digests may result either from intronic sequence in a single gene or from hybridization to distinct genes, all digests were reprobed with two nonoverlapping probes: (i) probe D9, an ≈500-bp portion of the P_{2U} receptor coding sequence (Fig. 4B, left lanes) or (ii) probe UT3P, a Kpn I fragment of HP2U, consisting of the 3′-most 350 bases of untranslated cDNA (Fig. 4B, right lanes). These probes recognized the same bands in DNA cut by EcoRI and Xba I (Fig. 4B), indicating that the multiple bands hybridizing to full-length HP2U cDNA in these digests do not result from a restriction site located in an intron of a single gene. In the BamHI digest,

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both probes hybridized strongly with the same large band, but one of the two smaller, less intensely hybridizing bands recognized by D9 was not recognized by UT3P. Consistent with the presence of a *Kpn* I site separating the sequences recognized by the tw probes, the *Kpn* I digest contained two different, strongly hybridizing bands, ne recognized by D9 and the other recognized by UT3P. However, a smaller, less intensely hybridizing band in the *Kpn* I digest was recognized by both D9 and UT3P, suggesting that a *Kpn* I site is not situated between the regions in this restriction fragment that hybridize with the probes. These findings suggest that the bands recognized by HP2U probes in restriction digests of human genomic DNA arise from two structurally similar genes.

In summary, HP2U represents a P$_2$-type nucleotide receptor isolated from human sources. The results of Northern analysis and the fact that identical cDNAs encoding the receptor were cloned independently from both airway and colonic epithelial cells attest to its wide distribution in human tissues. In some human tissues, the cloned cDNA hybridizes with multiple mRNA transcripts. The mechanism by which these transcripts are generated, including a possible correlation with the multiple hybridizing restriction fragments in human genomic DNA, remains to be definitively established. Heterologous expression of the receptor in one of the few cell lines that lack a response to extracellular nucleotides demonstrates that the clone encodes a protein possessing the functional properties of a UTP/ATP-selective, Ca^{2+}-mobilizing receptor coupled to G proteins and phospholipase effector enzymes—i.e., a P$_{2U}$ receptor. In addition, expression of HP2U in 1321N1 astrocytoma cells unexpectedly revealed autocrine feedback on the expressed receptor by nucleotides released from the cells. Further characterization of this phenomenon may provide important clues concerning mechanisms of cellular homeostasis. Most importantly, the availability of both the cloned human airway receptor and a specific, robust system for its expression will greatly facilitate identification of drugs that will for safety reasons most likely be based on pyrimidines (UTP) rather than purines (23, 24) for the treatment of CF and possibly other major human disorders.

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